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Induced Parasexual Processes in *Claviceps* sp. Strain SD58

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A homokaryotic, clavine alkaloid-producing strain of ergot, *Claviceps* sp. strain SD 58, was used in an attempt to demonstrate parasexuality. Genetically marked auxotrophic strains were produced by mutation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Protoplast fusion of pairs of unlike doubly auxotrophic strains and isolation of stable prototrophic fusion products were carried out. By growth of the fusion products on complete medium, selective pressure for prototrophy was removed and auxotrophic segregants were allowed to form. Analysis of these and recovery of segregants with nonleaky, non-parent-type combinations of auxotrophic characteristics has provided strong evidence that a parasexual cycle can function in *Claviceps* sp. strain SD 58. Preliminary work suggests that the genetics of ergot might be studied by mitotic analysis and that protoplast fusion and selection procedures might be useful for the enhancement of favorable characteristics in *Claviceps* strains.

Genetic studies of the ergot fungus and the application of these studies to alkaloid formation have received limited attention compared with genetic studies of other commercially and therapeutically important fermentation products such as the antibiotics and their producing organisms. This lack of attention can be attributed to the complex life cycle of *Claviceps* spp., which makes genetic recombination studies difficult.

At present, considering what is known about the biology of *Claviceps* spp., two possibilities exist for genetic recombination. One involves utilization of the sexual cycle of the fungus and subsequent meiotic recombination, and the second involves mitotic recombination through the parasexual cycle. In the first case, by using parasitic field cultivation through the host plant rye, Tudzynski and Esser (8) accomplished meiotic recombination of two different mutant strains of *Claviceps purpurea*. Approximately 50% parental types and 50% recombinants were recovered. Also using 10 wild-type strains of *C. purpurea*, the same group of investigators (9) isolated plasmids which were associated with the mitochondria and which were found to be linear and homologous to each other. In two of the strains, sequences homologous to the plasmids were integrated in the mitochondrial genomic DNA. It is still too early to determine whether these plasmids will be amenable to genetic recombination studies.

As a way to overcome the laborious and time-consuming processes involving field cultivation of ergot to obtain meiotic recombination, genetic recombination via the parasexual cycle has potential as a promising methodology for genetic studies on ergot and for production of strains of the fungus with enhanced abilities for the fermentative production of alkaloids. Parasexual reproduction involves heterokaryon formation by hyphal fusion, diploid formation by nuclear fusion in the vegetative hyphae, and mitotic segregation (crossing-over, nondisjunction, and haploidization). The last step of the process, mitotic segregation, provides a means for genetic recombination. Unfortunately, the parasexual cycle has never been conclusively demonstrated for *Claviceps* spp. A suggestion that mitotic recombination might have taken place in a cross between an adenine-requiring strain of *Claviceps paspali* and a phenylalanine auxotroph of *C. purpurea* has been made by Spalla and Marnati (6). Only phenylalanine-requiring progeny were obtained from the prototrophs produced in the cross. No adenine-requiring or recombinant types were recovered.

It was the purpose of this investigation to obtain evidence for the occurrence of the parasexual cycle in *Claviceps* sp. strain SD 58 by using doubly auxotrophic mutant strains, producing the heterokaryon through protoplast fusion, and examining auxotrophic segregants obtained from the fusion hybrid for genetic recombination.

MATERIALS AND METHODS

Strains and culture conditions. *Claviceps* sp. strain SD 58 (ATCC 26019, originally isolated from a sclerotium obtained from *Pennisetum typhoideum* Richard) was the parent from which the mutants used in these experiments were obtained. Auxotrophic mutants S-34 (Met^-) and 10-10 (Lys^-) were obtained from the collection of Srikrai and Robbers (7).

The following abbreviations are used to designate the phenotypic character of the auxotrophs: Ade^-, adenine requiring; Arg^-, arginine requiring; Cys^-, cysteine requiring; Leu^-, leucine requiring; Lys^-, lysine requiring; Met^-, methionine requiring; Nic^-, nicotinic acid requiring.

NL-406 complete medium (CM) in solid form was used for the maintenance of *Claviceps* sp. strain SD 58 wild-type strain, auxotrophic parental strains, and recombinants. It was also used as protoplast regeneration medium. NL-406 consisted of the following ingredients: mannitol, 50 g; sucrose, 50 g; succinic acid, 5.4 g; yeast extract, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.30 g; KH_2PO_4, 0.10 g; $ZnSO_4 \cdot 7H_2O$, 0.004 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and distilled H_2O to make 1 liter of medium. The pH was adjusted to 5.4 with NH_4OH. Solid medium for slants was made with 2.0% agar. For plates, 1.5% agar was used. Regeneration medium was made with 1.3% agar.

NL-406 minimal medium (MM) in solid form was used for initial auxotrophic selections, regeneration of prototrophic fusion hybrids, and maintenance of fusion hybrids. Its composition was the same as that of NL-406 CM except that yeast extract was omitted.

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Claviceps sp. strain SD 58 was maintained on CM agar slants. Liquid cultures, consisting of 100 ml of CM inoculated with a fragmented portion of the mycelium from a slant culture, were grown at 24°C and aerated by shaking at 220 rpm on a rotary shaker. After 7 days, the culture was homogenized in a Waring blender, and a 2-ml portion was transferred to fresh CM. Auxotrophic mutants were maintained and cultivated in the same manner.

Mutation and selection of auxotrophs. Mutant strains of *Claviceps* sp. strain SD 58 produced in earlier studies (7) were tested for nonleaky condition of their nutritionally deficient characters. Shake cultures were grown and used to inoculate CM agar plates in a regular pattern of 19 colonies per plate. When the colonies reached a diameter of 1 cm or more, they were replicated by hand in the same pattern on plates of MM. Those which failed to grow on MM after 2 weeks were considered to be nonleaky with respect to their auxotrophic characteristic. In cases in which a small amount of growth was encountered in most of the colonies on MM, the least leaky colony was subjected to subsequent similar selection procedures until a satisfactory nonleaky state was achieved. Nonleaky auxotrophs were subjected to a second mutagenic treatment by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Sigma Chemical Co., St. Louis, Mo.).

The Lys^- (strain 10-10) and Met^- (strain S-34) auxotrophic mutants or *Claviceps* sp. strain SD 58 were grown in submerged cultures for about 1 month to obtain sufficient conidia. The culture was filtered through a cloth filter in a Büchner funnel to separate the conidia from the mycelium. The filtrate was subjected to centrifugation at 4,500 × *g* for 10 min in an RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) with a Sorvall SS-34 rotor to concentrate the conidia. The conidia were suspended in 9 ml of sterile distilled water to which 1.0 ml of an aqueous solution containing 250 μg of NTG was added. The cells were incubated with the mutagen for 55 min at 24°C with shaking at 220 rpm. After this treatment, the conidia were concentrated by centrifugation and washed at least four times with sterile distilled water to remove NTG. They were then resuspended and quantitated by use of a hemacytometer. Serial dilutions were made, and aliquots were spread on plates of CM which were incubated at 24°C until colonies appeared.

Mutation of protoplasts was also carried out. Protoplasts were washed free of snail enzyme with sterile 0.7 M aqueous KCl solution and treated in the same manner as described for the mutation of conidia, except that 0.7 M KCl was used in all steps of the mutation procedure and 1 ml of an aqueous solution containing 1,000 μg of NTG in 9 ml of protoplast suspension was employed.

Colonies were replicated on CM and MM supplemented with the nutrient necessary to satisfy the first auxotrophic requirement. Those which grew on CM and not on MM were considered to have a second auxotrophic requirement and were characterized by being tested on diagnostic medium in a manner analogous to that of Holliday (2). All plates of the diagnostic media were supplemented with the nutrient needed to satisfy the first auxotrophic requirement.

The selection of double auxotrophs for nonleaky properties with respect to both characteristics was accomplished by using a procedure analogous to that used for single auxotrophs. (Colonies showing no growth on either of two plates, each supplemented with one of their nutritional requirements, were considered satisfactory.)

Formation and fusion of protoplasts. A double auxotroph was grown in 100 ml of liquid medium, homogenized, and transferred to new liquid medium. After 5 to 9 days of growth, the mycelium was collected by filtration through a coarse sintered-glass funnel. The mycelium was washed twice with 0.7 M KCl and suspended in 10 ml of the same solution to which was added 0.5 to 0.7 ml of glucuronidase-arylsulfatase from *Helix pomatia* (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The mixture was incubated with 100-rpm shaking at 29°C. After 2 to 3 h, the suspension was filtered through loosely packed glass wool to remove mycelial debris. The filtrate was centrifuged at approximately 4,500 × *g* for 10 min to recover the protoplasts. These were washed twice and finally suspended in the same solution for cell counting.

Equal quantities of protoplasts obtained from two different doubly auxotrophic strains were combined, mixed thoroughly, and centrifuged at 4,500 × *g* for 10 min. The supernatant was discarded, and 1 ml of prewarmed polyethylene glycol 6000 fusogen solution was added. The fusogen consisted of 30 g of polyethylene glycol 6000, 111 mg of $CaCl_2 \cdot 2H_2O$, 3.75 mg of glycine, and water to a final volume of 100 ml; the pH was adjusted to 7.5 with NaOH.

After mixing, the protoplast suspension was incubated at 30°C for 15 min. It was then diluted to 40 ml with 1.0 M $MgSO_4$ in 0.2 M NaOH–succinate buffer solution (pH 5.0). Serial dilutions of this solution were made with the same buffer. These were divided in 0.2-ml quantities onto plates of MM and CM regeneration medium. The number of colonies appearing on MM were compared with those growing on CM to obtain the fusion frequency. Colonies which grew on MM were transferred at least twice to new MM. Only those which retained their ability to grow on MM were considered to be prototrophic fusion hybrids. Haploidization of fusion hybrids was attempted by using two methods. In one, a fusion hybrid was transferred to a shake culture of CM supplemented with *p*-fluorophenylalanine (PFA; Sigma) in a 1/10,000 (wt/vol) ratio (4). In the other, the hybrid was transferred to a shake culture of CM without PFA. In each case, they were allowed to incubate at 24°C with shaking at 220 rpm for 2 weeks. They were transferred to fresh CM and allowed to incubate for 1 week. From these cultures, protoplasts were formed and were spread on CM regeneration plates. Colonies which formed on these plates were replicated in an orderly fashion on fresh CM plates.

Analysis of segregants. Colonies arising on the above-mentioned CM plates were replicated on CM and MM to test for auxotrophy. Those which failed to grow on MM were further characterized by being tested on four types of singly supplemented medium representing each auxotrophic requirement of the parental strains. They were also tested on doubly supplemented plates: six types of media represented all possible combinations of the four nutrients occurring in pairs. Tests were also run by using triply supplemented media, with four types of media representing all possible combinations of the four nutrients occurring in groups of three. The concentrations of nutrient supplements in the media were 75 mg/liter for methionine, lysine, leucine, and arginine and 15 mg/liter for adenine.

RESULTS

Mutagenic treatment of the single auxotrophic strain resulted in the formation of the following nonleaky double auxotrophs: Met^-/Ade^-, Met^-/Leu^-, Met^-/Lys^-, Met^-/Arg^-, Met^-/Nic^-, Lys^-/Leu^-, Lys^-/Cys^- or Met^-, Lys^-/Met^-, and Lys^-/Arg^-. On the basis of the ability for

TABLE 1. Auxotrophs arising from fusion hybrids

Type of auxotroph	No. of isolates from:				
	Met⁻/Ade⁻ × Lys⁻/Leu⁻			Met⁻/Arg⁻ × Lys⁻/Leu⁻	
	F_4 cultivated in CM + PFA	F_4 cultivated in CM[a]	F_1 cultivated in CM[b]	F_3 cultivated in CM + PFA[c]	F_3 cultivated in CM[d]
Single					
Lys⁻	4	1			
Leu⁻	1			14	1
Met⁻	4	29	17		8
Ade⁻	3	3	2		
Arg⁻				1	
Double					
Lys⁻/Leu⁻	5			1	1
Met⁻/Ade⁻	5	2			
Lys⁻/Ade⁻	1	7	2		
Met⁻/Lys⁻	2	1	1	1	
Met⁻/Leu⁻	1	3	1	1	
Leu⁻/Ade⁻	1				
Leu⁻/Arg⁻				5	
Triple					
Leu⁻/Lys⁻/Met⁻	1				
Quadruple					
Lys⁻/Met⁻/Ade⁻/Leu⁻	2	17	4		
Lys⁻/Met⁻/Arg⁻/Leu⁻				3	
Total colonies analyzed[e]	358 (8.4)	663 (9.5)	325 (8.3)	722 (3.6)	663 (1.5)

[a] The number of auxotrophs which tested consistently in all types of diagnostic media was as follows: 27 for single auxotrophs, 6 for double auxotrophs, and 17 for Lys⁻/Met⁻/Ade⁻/Leu⁻.
[b] The number of auxotrophs which tested consistently in all types of diagnostic media was as follows: 18 for single auxotrophs, 0 for double auxotrophs, and 4 for Lys⁻/Met⁻/Ade⁻/Leu⁻.
[c] The number of auxotrophs which tested consistently in all types of diagnostic media was as follows: 13 for single auxotrophs, 6 for double auxotrophs, and 3 for Lys⁻/Met⁻/Arg⁻/Leu⁻.
[d] The number of auxotrophs which tested consistently in all types of diagnostic media was as follows: 5 for single auxotrophs and 0 for double auxotrophs.
[e] Numbers in parentheses represent auxotrophs as a percentage of total number of colonies analyzed.

increased growth, Met⁻/Ade⁻, Met⁻/Arg⁻, and Lys⁻/Leu⁻ double auxotrophs were selected for fusion hybrid formation. The fusion of 2.5×10^9 protoplasts of each strain Lys⁻/Leu⁻ and Met⁻/Ade⁻ under optimal conditions resulted in a fusion frequency of 2.6%. Fusion of 5×10^9 protoplasts of each strain Lys⁻/Leu⁻ and Met⁻/Arg⁻ under optimal conditions occurred with a frequency of 4.3%.

Fusion hybrids with stable prototrophic characteristics (on MM) were considered for use in further studies. Since the fusion hybrids (like the double auxotrophic parent strains) lacked the ability to form conidia under these culture conditions, conidial size (5) could not be used as a means to detect diploidy. In the absence of direct evidence, successive reculturing of the hybrids in MM was considered to have provided an opportunity for nuclear fusion to occur.

Two Lys⁻/Leu⁻ × Met⁻/Ade⁻ fusion hybrids, F_1 and F_4, and one Lys⁻/Leu⁻ × Met⁻/Arg⁻ fusion hybrid, F_3, were each provided with an opportunity for haploidization. F_4 and F_3 were grown in both CM plus PFA and CM alone. F_1 was grown only in CM. For F_3 and F_4, it was possible to compare the effects of the use of CM plus PFA, a supposed haploidizing agent (4), with the mere removal of selective pressure for prototrophy (growth in CM alone).

Data from the analysis of segregants produced from F_1 and F_4 (Lys⁻/Leu⁻ × Met⁻/Ade⁻) hybrids and from F_3 (Lys⁻/Leu⁻ × Met⁻/Arg⁻) hybrids appear in Table 1. In all cases, auxotrophic segregants were recovered.

DISCUSSION

Mutagenic treatment of auxotrophic strains of *Claviceps* sp. strain SD 58 was attempted by using both conidia and protoplasts. The so-called conidia were actually a mixture of conidia and small mycelial fragments usually consisting of a single cell. In addition to the fact that mycelial fragments cannot easily be separated from conidia, this procedure suffers three drawbacks: (i) the singly auxotrophic strains require 30 days of growth to produce conidia; (ii) even after this time, few conidia are produced (as compared with the parent strain); and (iii) cells of the included mycelial fragments may contain more than one nucleus, which would impede detection of mutation events. The major advantage of using protoplasts in the mutation technique is that only 5 to 9 days of culturing are required before treatment, rather than the 30 days required to produce conidia.

The regeneration rate (ability to regain mycelial form) of protoplasts is quite low (less than 0.01%). After NTG treatment, only about 5% of these normally viable protoplasts are able to regenerate. Fusion frequencies were also on the order of 1 to 5%. For these reasons, it is necessary to carry out mutation and fusion procedures with 10^9 or more protoplasts.

The general life cycle of the subclass *Euascomycetidae*, which includes the genus *Claviceps*, has been described as being made up of mostly haploid structures. The only diploid

structure is the zygote which is formed by karyogamy in the sexual phase. (Some species of yeasts are exceptions to this rule[1].) With this in mind, it has been assumed without direct proof that the mycelial stage of *Claviceps* sp. strain SD 58, the structure being used in this experiment, is haploid and that fusion of two nuclei would produce a diploid.

In the case of haploidization, it can be seen from analysis of F_3 and F_4 segregants (Table 1) that the use of PFA had no appreciable effect in promoting segregation (haploidization). This is in marked contrast to the work of Lhoas (4), in which haploid segregants were produced from nearly all of the *Aspergillus niger* diploid conidia grown on PFA-supplemented medium; however, our result was consistent with the findings of Klinner et al. (3) that PFA did not cause a remarkable increase in the segregation frequencies of fusion hybrids of *Candida maltosa*.

Of major significance is the fact that some of the auxotrophic segregants did not test consistently in all of the different sets of diagnostic media, i.e., in singly, doubly, triply, and quadruply supplemented sets of plates. This may be because the colonies obtained after the segregation procedure were not homogeneous. This is believed to indicate that haploidization was not complete at the time of testing. In cases such as this, the diagnosis of auxotrophic characteristics was made from the results of the test on doubly supplemented plates. Consistency data are available for all segregants except those from the F_4 hybrid grown in CM plus PFA and are given in Table 1. These data for hybrids F_1 and F_4 grown in CM are based on agreement between the tests in singly and doubly supplemented media. For example, of 13 double auxotrophic segregants grown from the F_4 hybrid, only 6 completely failed to grow on any of the singly supplemented plates. For the F_3 hybrid, the data are based on agreement of all diagnostic medium tests.

In the absence of more direct evidence that the auxotrophic segregants recovered from the fusion hybrids have arisen by means of haploidization, one criterion used to determine whether a strain is haploid applies: the non-parent-type double auxotrophs, the triple auxotrophs, and the quadruple auxotrophs represent cases in which recessive markers originally introduced in repulsion in the parental diploid appear in coupling in the progeny. Unfortunately, the numbers of singly auxotrophic or prototrophic progeny that are actually haploid cannot be estimated (by the means at hand).

The production of non-parent-type auxotrophic segregants from the fusion hybrids is of greatest significance as an indication that parasexual processes have been induced in *Claviceps* sp. strain SD 58 by means of protoplast fusion. Among segregants arising from all three fusion hybrids, these recombinants occur at much higher frequency than is explainable by spontaneous mutation. The possibility of reversion of unstable auxotrophs to their parental type might have been considered for the single auxotrophs, except for their high frequency of occurrence.

Assuming that the genetic markers introduced in the parental strains by mutagenic treatment are recessive and that they are located on nuclear chromosomes, the observed evidence of recombination is likely to have been produced by parasexual means. The appearance of segregants bearing more than one auxotrophic character in combinations unlike either parent serves as the most convincing evidence that this is the case.

Colonies displaying recombinant-type auxotrophic characteristics were obtained from two different hybrids formed by the fusion of $Lys^-/Leu^- \times Met^-/Ade^-$ strains and a hybrid formed by the fusion of $Lys^-/Leu^- \times Met^-/Arg^-$ strains. Thus, the reproducibility of the result has been demonstrated. (The small sample size, the irregular distribution of types of recombinant segregants, and the procedure used to promote haploidization dictate that a conclusion concerning linkage relationships of these markers is inappropriate.)

To our knowledge, this preliminary work provides the first evidence that parasexual processes can be induced in a *Claviceps* sp. Hybridization by protoplast fusion and mitotic segregation have possible uses in the genetic analysis of this important group of drug-producing fungi. They perhaps have more immediate utility for the enhancement of favorable characteristics in the ergot fungus, since they may allow for the rapid crossing of strains.

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